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Kindlin-1 Is Required for RhoGTPase-Mediated Lamellipodia Formation in Keratinocytes

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Kindlin-1 is an epithelial-specific member of the novel kindlin protein family, which are regulators of integrin functions. Mutations in the gene that encodes Kindlin-1, *FERMT1 (KIND1)*, cause the Kindler syndrome (KS), a human disorder characterized by mucocutaneous fragility, progressive skin atrophy, ulcerative colitis, photosensitivity, and propensity to skin cancer. Our previous studies indicated that loss of kindlin-1 resulted in abnormalities associated with integrin functions, such as adhesion, proliferation, polarization, and motility of epidermal cells. Here, we disclosed novel *FERMT1* mutations in KS and used them, in combination with small-interfering RNA, protein, and imaging studies, to uncover new functions for kindlin-1 in keratinocytes and to discern the molecular pathology of KS. We show that kindlin-1 forms molecular complexes with β1 integrin, α-actinin, migfilin, and focal adhesion kinase and regulates cell shape and migration by controlling lamellipodia formation. Kindlin-1 governs these processes by signaling via Rho family GTPases, and it is required to maintain the pool of GTP-bound, active Rac1, RhoA and Cdc42, and the phosphorylation of their downstream effectors p21-activated kinase 1, LIM kinase, and cofilin. Loss of these kindlin-1 functions forms the biological basis for the epithelial cell fragility and atrophy in the pathology of KS. *(Am J Pathol 2009, 175:1442–1452; DOI: 10.2353/ajpath.2009.090203)*

Kindlins are a family of novel regulators of integrin signaling and cell-matrix adhesion,[1] which are causally linked to human genetic disorders. The family members, kindlin-1, kindlin-2, and kindlin-3 (also known as fermitin family homologs 1, -2, and -3), localize to integrin adhesion sites inside the cell and, together with talin, co-activate integrins to mediate outside-in signaling and to control cell behavior.[2–4] Kindlins are evolutionarily conserved multidomain proteins[1] that contain a hallmark C-terminal four point one band/ezrin/radixin/moesin (FERM) domain, but exhibit distinct tissue expression patterns: kindlin-1 is an epithelial-specific protein, kindlin-2 is widely expressed, and kindlin-3 is confined to the hematopoietic system.[5]

Pivotal information on kindlin functions has been gained from investigation of their defects in human diseases or mouse models.[2,3,6–8] In particular, the biological relevance of kindlin-1 was underlined by its association with Kindler syndrome (KS), a genetic skin disorder caused by mutations in the *FERMT1* (also known as *KIND1*) gene. Thus far, no human disease is known to be associated with genetic defects of kindlin-2,[9] but mutations in the kindlin-3 gene *FERMT3* were recently identified in rare leukocyte adhesion deficiency syndromes LAD-III and LAD-1/variant, with defective integrin activation in platelets, neutrophils, and lymphocytes.[7,10–13]

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Table 1. Patients with KS Investigated in This Study

Patient	Age, y	Mutation cDNA*	Mutation protein	Kindlin-1 expression	Mutation reference
1	7	IVS9_IVS11del/910G>T	P381RfsX36/E304X	Absent (Figure 1B)	6
2	27	1718 + 1G>A	C-terminal truncation	Reduced, truncated	35
3	40	910G>T	E304X	Absent	6
4	7	676dupC	Q226PfsX16	Reduced, truncated	15
5	28	456dupA	D153RfsX3	Absent	This study
6	62	328C>T	R110X	NA	16
7	10	328C>T	R110X	Absent	16
8	37	1365_1371 + 3del10	Frame shift	NA	This study
9	36	1209C>G	Y403X	Absent	This study
10	NA	1217dupA	N406KfsX1	NA	16

*If one mutation is mentioned, it is in a homozygous state. If two mutations are mentioned, the constellation is compound heterozygous; NA, not available.

KS is an intriguing human disorder affecting the skin, oral and urogenital mucosa, and the intestine.[14,15] It has an evolving phenotype that is not well understood: the clinical features change with advancing age and encompass congenital skin blistering, progressive poikiloderma, mucosal fragility, ulcerative colitis, photosensitivity, and propensity to epithelial cancer.[14,16] Epithelial fragility and atrophy are clinical hallmarks,[16] but despite rapid developments in understanding the genetic basis of KS, little is known about the molecular pathology and disease mechanisms underlying the clinical symptoms.

Morphologically, KS skin resembles the skin of mice with a keratinocyte-restricted β1 integrin knock out,[17] and the functional abnormalities of KS keratinocytes mirror those of perturbed integrin mediated processes.[6,17–19] This is in line with *in vitro* observations,[6,19] which suggest that kindlin-1 is required for keratinocyte proliferation, attachment to the extracellular matrix and motility. It may act via integrin activation and recruitment of specific molecules into the integrin-associated platforms, focal adhesions (FA), which allow force transmission. These processes require the integrity of the actin cytoskeleton and a fine-tuned regulation of its remodeling in response to different stimuli.[20] Depending on the stimulus and the signals it sets into motion, actin remodeling results in different structures. Such processes are governed by Rho GTPases, which induce either actin microfilaments that project filopodia, bundling of actin into fibers for efficient acto-myosin contraction during translocation of the cell body, or circumferential actin assembly and cell spreading.[21] Numerous actin-binding and other associated proteins act in a coordinated manner to respond to upstream signals and to remodel the actin cytoskeleton. Since such functions are important for cell survival, many molecules act in a redundant manner, as shown by the fact that more than 50 FA proteins have been identified.[22–25]

Here, we disclosed three novel *FERMT1* mutations and uncovered new physiological functions of the gene product, kindlin-1, by identifying novel ligands and showing that kindlin-1 modulates the cytoskeleton through Rho GTPase governed signaling processes in epithelial cells. These findings lay an essential basis for understanding the molecular pathology of KS and, consequently, for design of biologically valid therapeutic strategies for this incurable human disorder.

Materials and Methods

Patients with KS, Mutation Detection, Skin Samples, and Cell Cultures

Ten patients with KS (Table 1) were included in this study. Peripheral blood was obtained after informed consent. Genomic DNA extracted from peripheral lymphocytes was used for PCR amplification of the entire coding region and exon-intron boundaries of the *FERMT1* gene, as described.[26] The PCR products were processed for automated nucleotide sequencing in an ABI 3130XL genetic analyzer (ABI, Darmstadt, Germany). DNA sequences were compared with the reference sequence from the National Center for Biotechnology Information Entrez Nucleotide database (NM_017671) by using the Mutation Surveyor software (Softgenetics, State College, PA). The mutations were confirmed by re-sequencing of PCR products obtained from a second amplification reaction. Primary keratinocytes from normal control skin (normal human keratinocytes [NHK]) and KS skin were cultivated under serum-free conditions in defined keratinocyte growth medium (KGM; Invitrogen, Karlsruhe, Germany) as described.[6] Keratinocyte cell lines were derived from the skin of a healthy control and of patient 1 (coined KS-NM cells). These were immortalized with the HPV18 E6 and E7 genes.[27] In addition, for some experiments the epidermal HaCaT cell line was used (a kind gift of Dr. N. Fusenig, Das Deutsche Krebsforschungs zentrum, Heidelberg, Germany). The study was approved by the ethical committee of the University of Freiburg.

Primary Antibodies

In this study, primary antibodies against the following proteins were used for indirect immunoflurescence staining and/or immunoblotting: kindlin-1 (KS1 against a glutathione *S*-transferase [GST]-fusion protein spanning amino acids 541 to 674 of kindlin-1,[6] and KS4 against a peptide spanning amino acids 153-171 of kindlin-1[6]); α3 integrin (clone P1B5, Chemicon, Schwalbach, Germany); β1 integrin (clone JB1A, Chemicon); β1 integrin (clone HUTS-4, Chemicon); focal adhesion kinase (FAK) (clone 4.47, Upstate Biotechnology, Schwalbach, Germany);

phospho-FAK (Tyr397) (Biosource, Solingen, Germany); α-actinin (clone 0.T.02, Gene Tex, Irvine, CA); migfilin (clone 5E11, Abnova, Heidelberg, Deutschland); cofilin, phospho-cofilin (Ser3), LIM kinase (LIMK) 2, phospho-LIMK1 (Thr508)/LIMK2 (Thr505), p21-activated kinase 1 (PAK1), phospho-PAK1 (Thr423)/PAK2 (Thr402), (all from Cell Signaling Technology, Danvers, MA); E-cadherin (clone 36/E-cadherin, BD Biosciences, Heidelberg, Germany); collagen XVII;[28] vinculin (clone 7F9[29]); laminin α3 chain (clone BM-165,[30]); RhoA (clone 26C4, Santa Cruz, Santa Cruz, CA); Rac1 (clone 23A8, Upstate); Cdc42 (clone 44/CDC42, Transduction Laboratories, Heidelberg, Germany); and β tubulin (Biozol, Eching, Germany).

Immunofluorescence Microscopy

Skin cryosections were incubated with 0.1% bovine serum albumin/Tris-buffered saline for 30 minutes to block nonspecific binding sites and then incubated with primary antibodies overnight at 4°C or at room temperature.[6] Cells grown on coverslips were fixed with 2% paraformaldehyde in PBS for 15 minutes, washed three times with PBS, and treated for 1 minute with 0.1% Triton-X in PBS. Cells were then incubated at room temperature with primary antibodies for 1 hour or overnight, respectively. As secondary antibodies, Alexa anti-mouse or anti-rabbit IgG were used. Fibrillar actin was stained with phalloidin-Cy3 or -TRITC and nuclei with DAPI (Chemicon). Stained cells were observed with a confocal laser scanning microscope (LSM510, Carl Zeiss, Jena, Germany) or with epifluorescece microscope (Zeiss Axio Imager, Zeiss). Images were captured by using Zeiss internal software and processed by using ImageJ version 1.37g. The focal adhesion size and number were determined as described.[31]

Cell Adhesion and Proliferation Assays

For cell adhesion assays, tissue culture wells (96-well plates, Greiner, Frickenhausen, Germany) were coated with 2 ng/ml of laminin 332 (Sigma-Aldrich, Tauf kirchen, Germany) or 10 ng/ml of fibronectin (Sigma-Aldrich) at 4°C overnight. After saturation of the wells with 1% bovine serum albumin, equal numbers of cells were seeded. Cell were allowed to adhere for 1 hour at 37°C, and thereafter rinsed with PBS, fixed with 70% ethanol for 30 minutes at room temperature, and stained with 0.5% crystal violet for 15 minutes. Adherent cells were quantified by measuring the OD at 540 nm with an Infinite 200 microtiter plate reader (Tecan, Austria). Data are the means of eight measurements.

Cell proliferation was assessed by using the (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) cell proliferation assay (American Type Culture Collection, Manassas, VA). Equal numbers cells treated with kindlin-1 specific or control small-interfering (siRNA) were seeded in triplicate in 96-well plates. At the indicated time points, the cells were stained by incubation with MTT; subsequently, the detergent reagent was added to each well, and the cell numbers were quantified by measuring the absorbance at 570 nm on an Infinite 200 microtiter plate reader (Tecan, Austria).

Cell Motility Assays

In vitro wound healing assays were performed as described.[6] The scratched areas were photographed with a digital compact camera C-7070 (Olympus, Hamburg, Germany) immediately after wounding and then every 2 hours during 12 hours. Time-lapse video microscopy was used to monitor migration of individual keratinocytes. The cells were seeded in KGM with additives, and after reaching 30 to 40% confluence, cell movements in 5% CO_2 at 37°C were recorded in a BioStation IM (Nikon, Düsseldorf, Germany). Phase contrast photographs were automatically captured every 5 minutes for 3 hours. To determine the percentage of polarized, fan-shaped cells,[32] keratinocytes at 30 to 40% confluence were photographed with the digital compact camera C-7070 (Olympus). Phase contrast images of three separate fields containing approximately 100 to 200 cells were obtained for each culture plate. For a cell to be scored as polarized, it had to have the following properties: (a) a phase-bright retracted rear, which extends across the cell diameter; (b) the nucleus polarized to the rear of the cell; (c) a single, large lamellipodium that extends around the remaining circumference of the cell.[32]

siRNA Treatment

HaCaT cells were transfected with siRNA duplexes (Eurogentec, Liège, Belgium) specific to kindlin-1 (sequence 1: 5′-GAAGUUACUACCAAAAGCU-3′; sequence 2: 5′-ACUUGCAGAUAAUCUCAAA-3′) and with an irrelevant control siRNA duplex, using HiPerFect (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. At different time points after transfection, expression of kindlin-1 was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting.

Protein Extraction and Immunoblotting

Cell layers were lysed and extracted in a buffer containing 1% Nonidet P-40, 20 mmol/L Tris-Cl (pH 7.5), 100 mmol/L NaCl, 4 mmol/L EDTA, 1 mmol/L Pefabloc, protease inhibitor cocktail set III (Calbiochem), and phosphatase inhibitor cocktail I (Sigma) for 30 minutes at 4°C. The lysates were centrifuged at 14,000 rpm for 30 minutes at 4°C; the supernatants were assayed for protein concentration by using the DC Protein Assay (Bio-Rad, München, Germany). For immunoblotting, the proteins were separated on 10% or 12% SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose membranes. The blots were incubated with primary antibodies overnight at 4°C, followed by incubation with alkaline phosphatase-linked anti-rabbit or anti-mouse IgG (both from Sigma), or horseradish peroxidase-labeled anti-mouse IgG (Bio-Rad) for 1 hour at room temperature. For detection of phosphorylated proteins, membranes were incubated with antibodies diluted in 5% (w/v) bovine serum albumin in Tris-buffered saline, 0.1% Tween-20 at 4°C overnight. Immunoblotting with antibodies to β tubulin was used to control loading.

The visualization was performed with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate in detection buffer or with the ECL Plus System (Amersham, Freiburg, Germany). Scanning densitometry of band intensity was performed with the Gel-Pro Express 4.0 software (Media Cybernetics Inc., Bethesda, MD).

Co-Immunoprecipitations

Co-immunoprecipitation (Co-IP) experiments were performed with two different domain-specific kindlin-1 antibodies.[6] For Co-IP with the affinity purified antibody KS4 to N-terminal kindlin-1 (amino acids 153 to 171),[6] confluent NHK were extracted with 1% 3[(3-cholamidopropyl) dimethylammonio]–propanesulfonic acid in PBS supplemented with protease inhibitor cocktail set III (Calbiochem) and phosphatase inhibitor cocktail I (Sigma) for 15 minutes at 4°C. For co-immunoprecipitation with the antibody KS1 to C-terminal kindlin-1 (amino acids 541 to 674),[6] confluent NHK were extracted in lysis buffer containing 1% Nonidet P-40, 25 mmol/L Tris-Cl (pH 7), 100 mmol/L NaCl, and the same inhibitors as above. Two hundred μl of the sera were covalently bound either to CNBr Sepharose, or to Protein G Sepharose (GE Health Care, München, Germany). One hundred μl of each antibody-Sepharose beads were incubated with 150 μl of cell lysate overnight in Micro Bio-Spin Chromatography columns (Bio-Rad) under constant shaking at 4°C. After two washing steps with the lysis buffer, the proteins were eluted with 100 μl of 0.1 M citric acid, pH 3.2, and the eluates were neutralized with 1 M Tris-HCl. The immunoprecipitates were analyzed by immunoblotting with antibodies to kindlin-1, β1 integrin, α-actinin, FAK migfilin, and collagen XVII. Reverse experiments were performed by using antibodies to α-actinin, FAK, and migfilin for Co-IP and to kindlin-1 for immunoblotting. As negative controls, irrelevant rabbit IgG were used for Co-IP.

Rho GTPase Pull-Down Assays

GTPase pull-down assays were performed as described.[33] Briefly, subconfluent cells were lysed and GTP-bound, active GTPases were pulled down by either GST- P21-Rho-binding domain fusion protein with the Cdc42- and Rac1-binding region of PAK-1B, or the GST-ras-binding domain fusion protein with the RhoA-binding region of rhotekin. The cell lysates and the pull-down fractions were separated by SDS-polyacrylamide gel electrophoresis on 12% polyacrylamide gels under reducing conditions. The proteins were transferred to nitrocellulose membranes and incubated with monoclonal antibodies to either RhoA (Santa Cruz), Rac1 (Upstate) or Cdc42 (Transduction Laboratories), followed by secondary horseradish peroxidase-conjugated antibodies (DAKO Glostrup, Denmark). The signals were visualized by using enhanced chemiluminescence (Amersham Biosciences). Band intensities were quantified with ImageJ software and the relative amount of active, GTP-bound GTPase was normalized to the total content of GTPase in the lysate.

Results

Phenotypic Anomalies of Skin and Mucosa Are Associated with Altered Keratinocyte Shape in KS

All patients had homozygous or compound heterozygous *FERMT1* mutations, which led to absence of kindlin-1 protein, or to residual expression, about 5% of normal, of C-terminally truncated kindlin-1 (Table 1). Three of the mutations are novel and lead to premature termination codons: c.456dupA, c.1365_1371 + 3del10, and c.1209C>G. The deletion c.1365_1371 + 3del10 involves the last seven nucleotides of exon 11 and the first three adjacent intronic nucleotides. The consequence on protein level is predicted to be a frame shift and a premature stop codon at the very next position. Phenotypically, the main symptom in younger patients was skin blistering, while adult patients suffered mainly from consequences of skin atrophy (Figure 1A), pigment anomalies (Figure 1B) and of ocular, esophageal, anal, or genital mucosal erosions with subsequent scarring (Figure 1C). KS skin was analyzed microscopically to disclose the consequences of kindlin-1 deficiency *in vivo*. All patients had no or strongly attenuated kindlin-1 protein in the skin (Table 1, Figure 1, D and E). The morphology of epidermal keratinocytes was visualized by using immunofluorescence staining with an antibody to α3 integrin, a transmembrane protein distributed along the entire plasma membrane of basal keratinocytes. In normal human epidermis, basal keratinocytes have a relatively uniform columnar shape (Figure 1F, Table 2). In contrast, in KS skin basal keratinocytes display significant shape variability, ie, elongated keratinocytes alternate with flat, irregularly shaped cells (Figure 1G, Table 2).

Kindlin-1 Is Required for Lamellipodia Formation and for Proper Distribution of Focal Adhesions In Vitro

The polymorphic appearance of basal keratinocytes in KS skin is mirrored by the irregular morphology of primary KS keratinocytes *in vitro* (Figure 2A). We investigated primary cells from three patients with KS and three different controls. The cell shape changes were present in all patients, in all passages used for experiments (passages 2 to 4). KS keratinocytes display elongated shapes, multiple cellular protrusions of different orientation and populated by actin microfilaments, and a thin network of cortical actin. These features are in contrast to the typical polygonal morphology and prominent network of cortical actin observed in NHK (Figure 2A, Table 2). While more than 45% of NHK in a defined, serum-free and low calcium, growth medium had a polygonal, fan-shaped appearance, only 12% of KS cells had this morphology (Figure 2B), the difference being highly significant ($P < 0.0001$).

To examine whether the cytoskeletal and cell shape alterations observed in KS cells associate with abnormalities in the subcellular distribution of proteins, FAs were

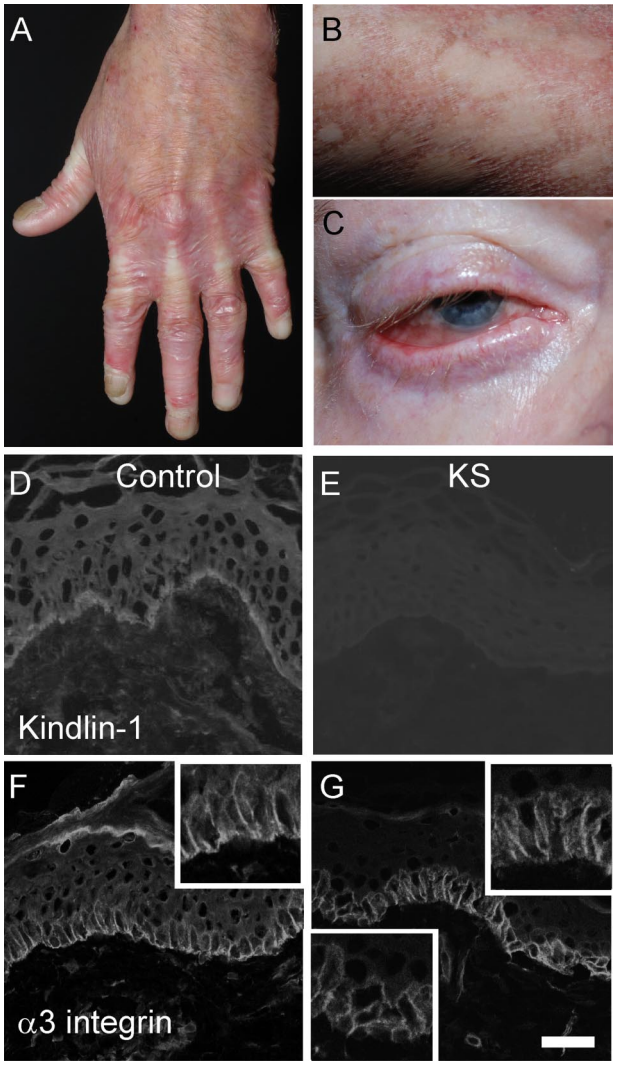


Figure 1. Macroscopic and microscopic anomalies in KS skin. Clinically, skin atrophy, pigment anomalies, and mucosal erosions are major symptoms of KS. **A:** Note the pronounced atrophy of the skin on the dorsal aspects of the hands, the webbing of the fingers, and the nail dystrophy in the 28-year-old patient 5. **B:** Poikilodermatic lesions consisting of hypo- and hyperpigmented patches, atrophy, and telangiectasia on the arm of patient 5. **C:** Ectropion and conjunctivitis are major symptoms of patient 6 at 62 years of age. **D** and **E:** Indirect immunofluorescence staining of control and KS skin with kindlin-1 antibodies. In control skin, kindlin-1 exhibited a linear distribution at the dermal-epidermal junction (**D**), but remained negative in KS skin (**E**). Morphologically, loss of kindlin-1 is associated with abnormal shape of basal keratinocytes. The cell shape was visualized with antibody staining of $\alpha 3$ integrin, which is localized at the plasma membrane in the entire periphery of basal keratinocytes (**F** and **G**). In control skin (**F**), basal keratinocytes exhibited a cuboidal or columnar shape (**inset**), whereas in KS skin **(G)** they had a variable appearance, with stretches of vertically elongated cells alternating with stretches of flattened, irregular cells (insets). **Insets** show twofold magnifications. Scale bar for **D** and **G** = 20 μm.

assessed by vinculin staining (Figure 2A). The size and number of FAs were not significantly different in NHK and KS keratinocytes, the mean FA area in NHK being 1.41 μm^2 versus 1.33 μm^2 in KS cells, and the average number of FA in NHK being 61.6 versus 58.2 in KS cells (Table 2). In contrast, the distribution pattern of FA within the cells clearly differed. In NHK, robust FA were localized at the entire cell periphery, as well as all over the ventral surface of the cell body, whereas in KS cells, FA were restricted to the cell periphery, preferentially to cellular protrusions (Figure 2A). Determination of the mean distance of FA to the cell periphery revealed a statistically highly significant shift in the distribution of FA to a more peripheral location in KS cells (Figure 2C). The distance in NHK was 6.72 $\pm$ 5.52 μm, and in KS cells 4.41 $\pm$ 3.67 μm ($P < 0.0001$). The above studies were performed with primary human keratinocytes isolated from the skin of healthy controls or individuals with KS. To verify that the consequences of loss of kindlin-1 were not limited to primary cells, we established an immortalized KS cell line, designated KS-NM, and showed that it preserves the main characteristics of the primary KS cells (Figure 3). Compared with immortalized control keratinocytes (Co), KS-NM cells had a polymorphic, predominantly elongated shape (Figure 3, A and B). The kindlin-1-deficient cells were significantly smaller than controls ($P < 0.0001$), with a mean area of 1861 $\pm$ 762 μm^2 for KS-NM cells, and of 2692 $\pm$ 833 μm^2 for immortalized control keratinocytes.

Table 2. Characteristics of Primary NHK and KS Keratinocytes

Cell	NHK	KS	p (*t*-test)
FA size, μm^2	1.41	1.33	0.27
Number of FA per cell, mean	61.6	58.2	0.75
Distance of FA to cell periphery, mean, μm	6.72	4.41	<0.0001
Polygonal cells, %	45	12	<0.0001

To prove that lack of kindlin-1 is directly responsible for the altered cell shape, we used siRNA to down-regulate its expression in HaCaT cells. With two different siRNAs, about 80 to 90% reduction of kindlin-1 levels was obtained 72 to 120 hours after transfection (Figure 4A). The down-regulation generated clear alterations in cell morphology. Instead of the uniform polygonal shape observed with the cells treated with a control siRNA, cells treated with kindlin-1 - specific siRNAs exhibited irregular, elongated shapes (Figure 4B). Their area was significantly smaller than of the cells treated with control siRNA (658 $\pm$ 204 μm^2 versus 826 $\pm$ 243 μm^2, $P < 0.0013$). The proliferation rate of kindlin-1 deficient HaCaT cells was about 37% lower than that of control cells ($P < 0.0001$). The adhesion to laminin 332 and fibronectin was also significantly reduced compared with control cells, by 40% and 44%, respectively (Figure 4C).

These observations demonstrate that loss of kindlin-1 in keratinocytes strongly affects cell morphology. It allows formation of FA, but leads to absence of FA at the basal surface of the cell bodies, suggesting abnormalities in integrin-dependent signaling pathways.

Lack of Kindlin-1 Alters Cell Migration

To further explore the functional consequences of loss of kindlin-1, cell migration was assessed in after scratch-wounding of confluent cell monolayers. NHK migrated into the wound area as a contiguous sheet and maintained their cell-cell contacts as they moved. In contrast, KS cultures lost the integrity of the wound edge, and

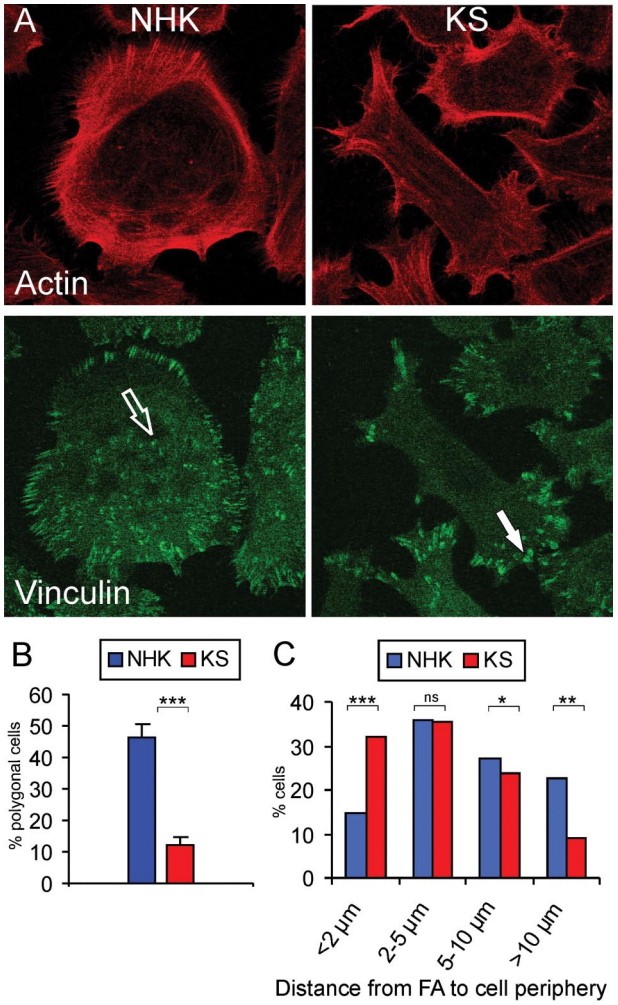


Figure 2. Primary kindlin-1 negative keratinocytes exhibit abnormal shape, lack of a large lamellipodium, and abnormal distribution of FA *in vitro*. **A:** Primary NHK and KS keratinocytes were stained with Cy3-conjugated phalloidin to visualize the actin cytoskeleton (red, **upper panels**) and with vinculin antibodies to demonstrate FA (green, **lower panels**). Corresponding to their elongated and multipolar shape, KS cells had reduced circumferential cortical actin, and concentration of the FA to cellular extensions (**lower right panel, arrow**). In NHK, FA were distributed along the entire cell periphery and between the cell body and culture dish (**lower left panel**, **open arrow**). In contrast, ventral focal adhesions, also designated as focal complexes were rarely detected in KS cells. For all panels the scale bar = 20 μm. **B:** The cells displaying a polarized, fan-shaped (presence of a large lamellipodium) morphology were counted. For each cell population, more than 300 cells in three different culture dishes were analyzed. For a cell to be scored as polarized, it had to have the following properties: (a) a phase-bright retracted rear, which extends across the cell diameter; (b) the nucleus polarized to the rear of the cell; and (c) a lamellipodium that extends around the remaining circumference of the cell.[32] The graph shows that about half of NHK (blue) displayed such a conformation, but only 12% of KS cells (red; $P < 0.0001$). **C:** The position of FAs relative to the cell periphery was quantified in NHK (blue) and KS keratinocytes (red) by using ImageJ software. The columns represent the percentage of FA located at a distance of less than 2 μm, 2 to 5 μm, 5 to 10 μm, and over 10 μm from the cell periphery. For each cell type, more than 1000 FA were analyzed. The percentage of FA located at less than 2 μm from the cell periphery was significantly higher in KS cells than in NHK ($P < 0.0001$), while the percentage of FA distributed at more than 10 μm from the cell periphery was significantly higher in NHK ($P < 0.01$).

individual cells scattered randomly into the wound area (Figure 5A).

Time-lapse microscopy was used to observe migration of individual keratinocytes cultured to 30 to 40% confluence. The majority of the KS cells did not migrate by extending a large, unique polarized lamellipodium, like NHK. Instead, KS cells extended several protrusions ending with small, perhaps competing, lamellae (Figure 5B). The average lamellar area was significantly smaller ($P < 0.001$) in KS cells (861 $\pm$ 267 μm^2) compared with NHK (1554 $\pm$ 773 μm^2). Since keratinocytes deposit laminin 332 as they migrate,[32] we assessed the pattern of laminin 332 deposition by KS keratinocytes. Cells seeded on coverslips were allowed to adhere and grow for 2 days to a density of about 30%, then fixed and stained with antibodies to the laminin α3 chain, phalloidin-conjugated dye, and DAPI to visualize the cell bodies and nuclei, respectively. NHK left behind a laminin 332 positive track as they migrated (Figure 5C). In contrast, the symmetrical deposition of laminin 332 beneath the KS cell bodies reflected the abnormal migratory behavior of these cells, ie, that they do not translocate the cell bodies significantly.

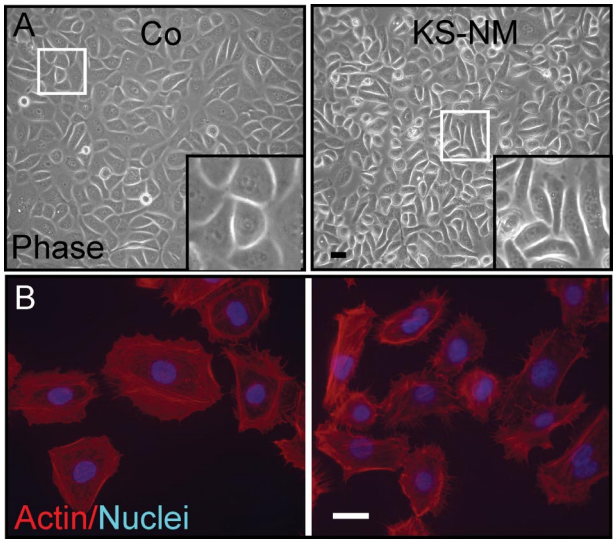


Figure 3. Immortalized kindlin-1 null KS-NM cells display shape abnormalities. **A:** Immortalized NHK (Co) and KS-NM cells were grown to confluence in KGM under serum-free conditions. Phase-contrast microscopy demonstrates the polygonal shape of control cells (**inset**) and the predominantly elongated shape of KS-NM cells (**inset**). Insets show 2.5-fold magnifications of the marked areas. **B:** Immortalized NHK (Co) and KS-NM cells were grown to 30 to 40% confluence and stained with phalloidin-TRITC to visualize fibrillar actin and with DAPI to visualize the nuclei. Compared with controls, KS-NM cells are clearly smaller, containing more cellular protrusions and extensions, occasionally ending with small lamellipodia. Scale bars = 20 μm.

Kindlin-1 Interactions in Molecular Complexes

Recruitment of kindlin-1 into molecular complexes with β1 integrin[19] and its binding to kindlin-2 and migfilin[9] do not explain all alterations observed in KS. Therefore, we searched for further binding partners in FA to explore the structural basis of kindlin-1 functions. Co-IP experiments with two different domain-specific kindlin-1 antibodies identified β1 integrin, α-actinin, migfilin, and FAK as binding ligands of kindlin-1 (Figure 6). Vice versa, when integrin β1, α-actinin, or FAK antibodies were used for immunoprecipitation, both phosphorylated and unphosphorylated kindlin-1 was present in the complexes, thus defining kindlin-1

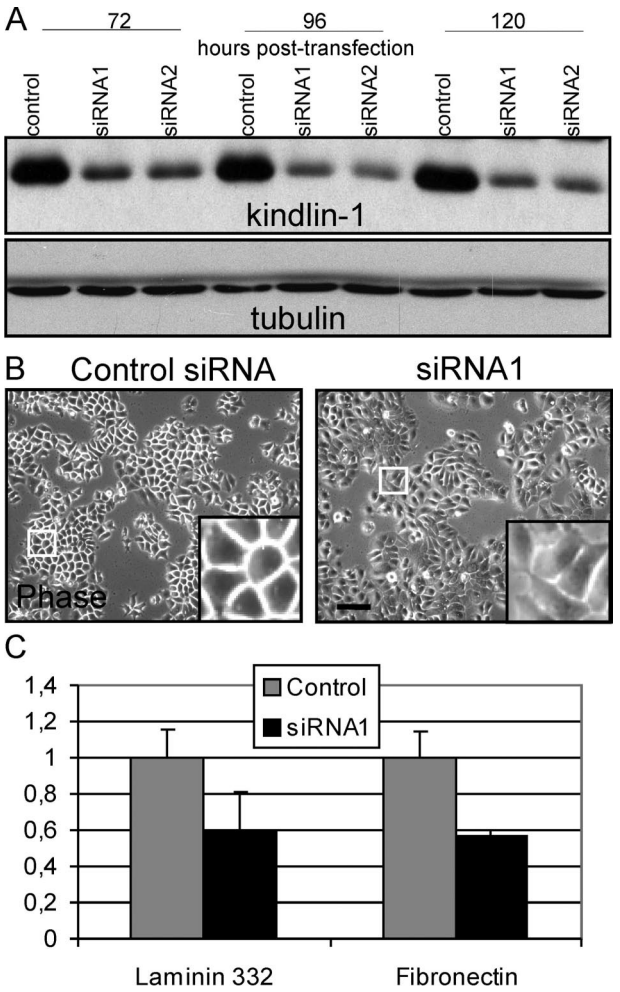


Figure 4. Down-regulation of kindlin-1 with siRNA alters cell shape. **A:** HaCaT cells were transfected with control siRNA (control) or two different *FERMT1* siRNAs (siRNA1, siRNA2), and analyzed after 72, 96, and 120 hours by immunoblotting with kindlin-1 antibodies. Treatment with siRNA1 and siRNA2 reduced kindlin-1 expression by about 80%. Immunoblotting with antibodies to β tubulin was used to control loading. **B:** Phase contrast micrographs show that after treatment with kindlin-1 specific siRNAs, HaCaT cells (**right panel** and **inset**) became elongated and lost the typical polygonal form seen in controls (**left panel** and **inset**). **Insets** represent 3.6 magnifications of the marked areas. Scale bar = 20 μm. **C:** To assess cell adhesion, equal amounts of control and kindlin-1 specific siRNA treated HaCaT cells were plated for 1 hour on surfaces coated with laminin 332 or fibronectin, washed, fixed, and stained with crystal violet. The adhesion of kindlin-1 knockdown cells was reduced compared with control cells, by 40% and 44%, respectively.

as an integral component of the focal adhesions in epidermal keratinocytes. The total amount of β1 integrin, migfilin, α-actinin, kindlin-2, and FAK were similar in primary KS cells and NHK, as well as in the immortalized KS-NM and NHK cell lines (Supplementary Figure 1A, see *http://ajp.amjpathol.org*), suggesting that loss of kindlin-1 does not interfere with the expression, or cause up-regulation of these binding partners. The expression and distribution of these proteins in KS skin was comparable with the control skin, although a slight reduction of α-actinin and FAK in the basal layer could be observed (Supplementary Figure 1B, see *http://ajp.amjpathol.org*). Similarly, *in vitro*, a decreased level of phospho-FAK (Tyr397) was present in KS cells, compared with control cells (Supplementary Figure 1A, see *http://ajp.amjpathol.org*).

Kindlin-1 Is Required for Activation of Rho GTPases in Keratinocytes

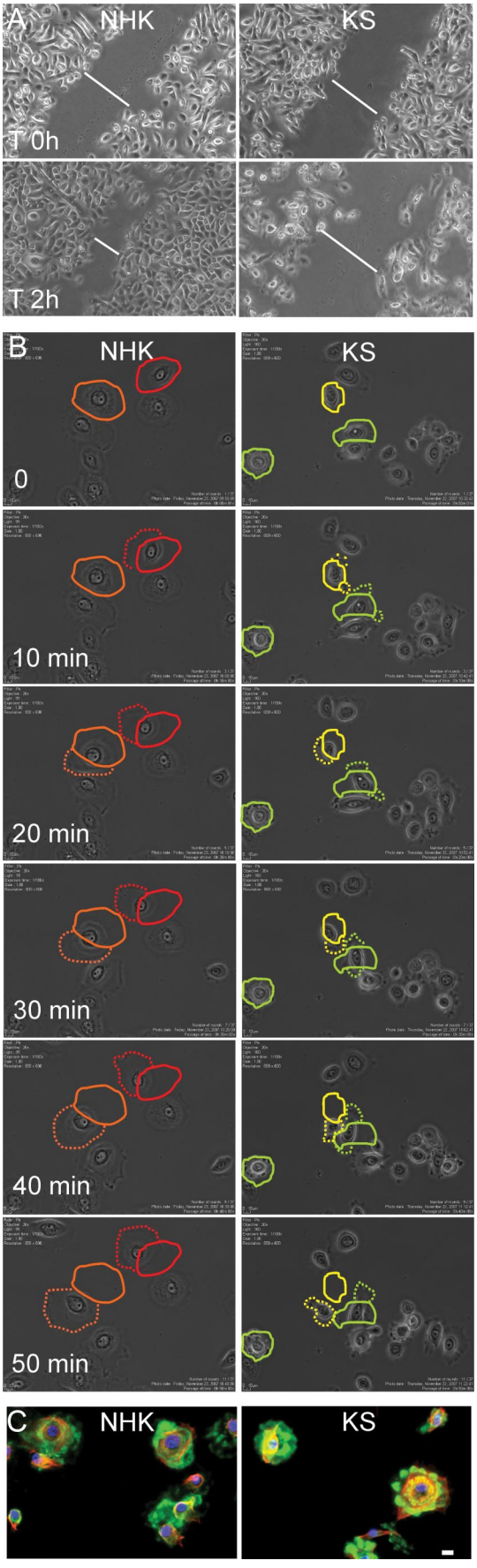
Rac1, RhoA, and Cdc42, three members of the Rho family of small GTPases control a signal transduction pathway linking cell surface receptors to the assembly and disassembly of the actin cytoskeleton and of associated integrin adhesion complexes.[21] They are involved in many cellular functions, including the formation of lamellipodia and cell migration. Therefore, we examined the effects of the absence of kindlin-1 on Rho GTPase activity. The GTP-bound (active) forms of Rac1 and RhoA were drastically diminished in KS keratinocytes, representing about 10% and 25%, respectively, of control levels (Figure 7A). The GTP-bound (active) forms of Cdc42 were also reduced, although to a lesser extent representing about 50% of controls (Figure 7A). To examine whether reducing the pool of GTP-bound Rho GTPases in KS cells had further consequences, we analyzed downstream targets. The phosphorylated active forms of PAK1/PAK2 and their substrate LIMK1/LIMK2, specific downstream targets of Cdc42 and Rac1, were reduced in KS cells. The levels of phosphorylated PAK1/PAK2 were decreased by 66% and those of phosphorylated LIMK1/LIMK2 by 45%, as compared with controls. Subsequently, also, cofilin phosphorylation, a process mediated in part by LIMK, was reduced by about 40% in KS cells (Figure 7B).

Taken together, these results indicate that in the absence of kindlin-1, the functionality of β1 integrin associated complexes is disturbed affecting the propagation of signal transduction events that regulate cell migration and polarity through small Rho GTPases (Figure 8).

Discussion

Here, we deliver genetic and biological evidence for the involvement of kindlin-1 in integrin-mediated outside-in signaling in keratinocytes. As a basis for delineating the molecular mechanisms of kindlin action, the genotypes and phenotypes of 10 individuals with KS and mutations in the *FERMT1* gene encoding kindlin-1 were evaluated, and three novel mutations were disclosed, all of which led to absence of kindlin-1 in the tissues. These data expand the mutation data base of kindlin gene defects and help define the link between members of the novel kindlin protein family and human disease.

Our previous work showed that β1 integrin-mediated adhesion of keratinocytes is defective in absence of kindlin-1, although the expression of β1 integrin itself and talin were not changed.[6] The role of kindlin-1 in integrin activation has also been demonstrated in human and murine colon epithelial cells.[8,15] Similarly to kindlin-2 and kindlin-3, recently identified as co-activators of integrins,[2,3] kindlin-1 possesses the phosphotyrosine-binding domain subdomain, and binds directly to the intracellular tail of



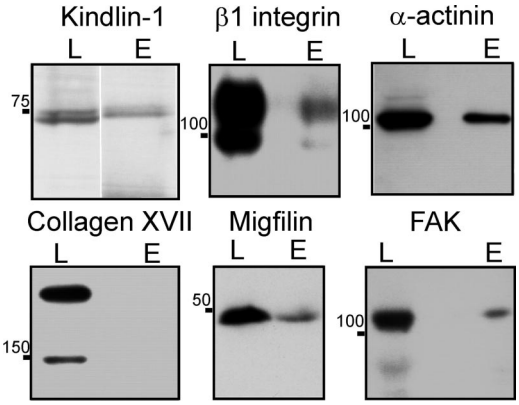


Figure 6. Kindlin-1 binds β1 integrin, α-actinin, migfilin, and FAK. NHK lysates (L) used for immunoprecipitation with the kindlin-1 antibody KS1 and the corresponding elution fractions (E) were analyzed by immunoblotting with antibodies to kindlin-1, β1 integrin, α-actinin, collagen XVII, migfilin, and FAK. β1 integrin, α-actinin, migfilin, and FAK co-precipitated with kindlin-1, but not collagen XVII. Numbers on the left of each panel represent the migration position of the molecular weight standards in kDa.

β1 and β3 integrins,[19,34] suggesting that kindlin-1 is directly involved in β1 integrin activation in keratinocytes. This prediction is also supported by genotype-phenotype correlations in KS. Several patients reported in this study (Table 1) and elsewhere[35,36] who carry *FERMT1* truncation mutations leading to ablation of the PTB domain, had severe phenotypes similar to those caused by complete loss-of-function mutations. However, Ussar et al[8] showed that no significant decrease in integrin activation was detected in freshly isolated keratinocytes from the kindlin-1 knockout mouse.

The present *in vitro* data indicate that lack of kindlin-1 is compatible with FA formation at least in the periphery of keratinocytes, where integrins are able to nucleate the assembly of FA. It is possible that this process can proceed because another molecule, for example kindlin-2, compensates for lack of kindlin-1 functions. However, changes in the expression levels or the gross distribution of kindlin-2 in KS skin have not been found (authors' unpublished observation),[9,8] although more subtle, hitherto undetected modifications still remain a possibility. Another compensatory molecule could be β6 integrin, since the elongated, "rod-like," and peripherally localized FA observed in KS cells resemble those observed in β1

Figure 5. Altered migration of KS cells. **A:** *In vitro* wound closure assays were photographed every 2 hours for 12 hours.[6] Here, representative wound edges at time point 0 and 2 hours are shown. Note that NHK migrate as a coherent front and close the wound faster than KS cells, which move individually and in a scattered manner. **B:** NHK and KS cells were allowed to attach and grow to 30 to 40% confluence in KGM. Time lapse images were captured every 5 minutes for 3 hours. For both cell types, representative micrographs at time points (T) 0, 10, 20, 30, 40, and 50 minutes are shown. At T 0, the border of two NHK and three KS cells is marked with a continuous line (red and orange for NHK, and yellow and green for KS); at the next time points the cell contours are depicted with a dotted line. Note that NHK extend large lamellipodia and move forwards, while retracting the body at the opposite pole. In contrast, KS cells extend multiple protrusions and retract their bodies in different directions. **C:** Primary NHK and KS cells were plated on glass coverslips, allowed to adhere and grow for 24 hours and stained with antibodies to the laminin α3 chain (green), Cy3-conjugated phalloidin (red), and DAPI (blue). Note the scattered deposition of laminin indicating the migration tracks of the control cells, and the symmetric and regular deposition of laminin restricted beneath the KS cell bodies, which had not moved. Scale bar = 20 μm.

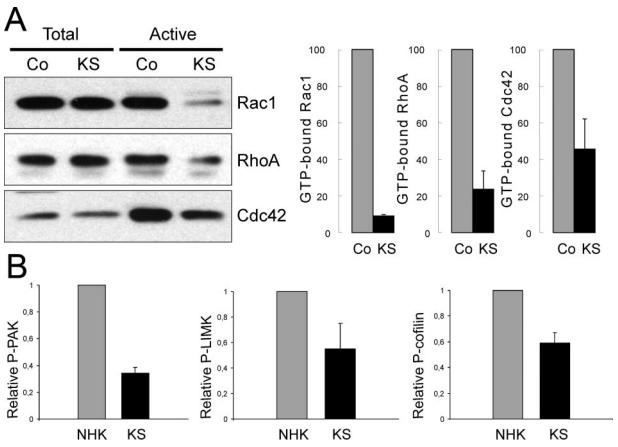


Figure 7. Reduction in the GTP-bound, active Rho GTPases, and reduced phosphorylation of their downstream effectors in KS keratinocytes. **A:** GTP-bound, active forms of the Rho GTPases were precipitated from lysates of immortalized NHK (Co) and KS-NM (KS) cells. The **left panel** shows a representative immunoblot of whole cell lysates (Total) and of precipitated GTP-bound (Active) Rac1, RhoA, and Cdc42. On the **right**, the columns represent the relative amounts of GTP-bound Rac1, RhoA, and Cdc42. For quantification, band intensities were measured on blots obtained in three independent experiments and normalized to total GTPase levels. In each experiment, the Rho GTPase levels for control cells (Co) were set as 100 and those for KS cells were calculated as a percentage of controls. Mean average and the SD are shown. **B:** Primary NHK and KS keratinocytes were grown to 80 to 90% confluence, lysed, and analyzed by immunoblotting with phospho-specific and nonphospho-specific antibodies to PAK, LIMK, and cofilin. The graphs show the relative densities of the phosphoprotein bands, normalized to the amount of total proteins, and the SD. The gray columns correspond to NHK and the black columns to KS cells. As a consequence of kindlin-1 deficiency, the phosphorylated form of PAK was decreased by 66%, the phosphorylated form of LIMK by 45%, and that of cofilin by 41%. The results represent three independent experiments.

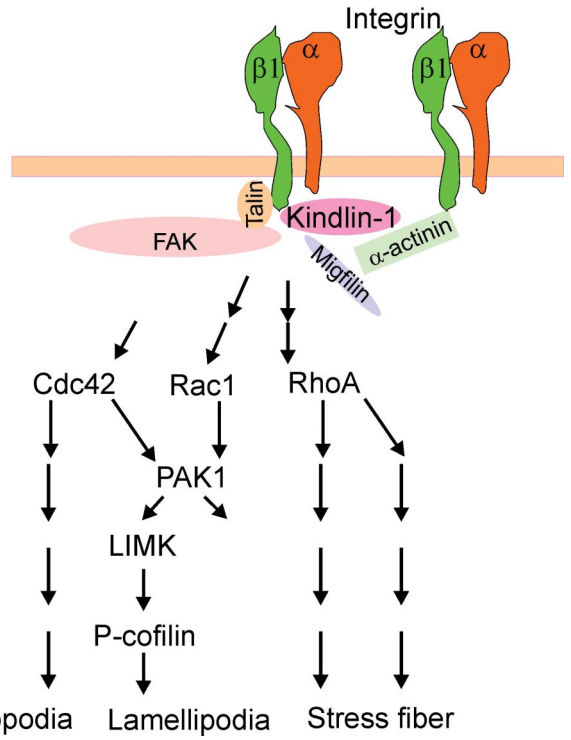


Figure 8. Model for the functional role of kindlin-1 in keratinocytes. Kindlin-1 forms complexes with the β1 integrin subunit, α-actinin, FAK, and migfilin. RhoGTPases Rac1, Cdc42, and RhoA are activated and regulate lamellipodia protrusions and membrane ruffles, filopodial extensions, and stress fibers through numerous effectors.

null keratinocytes, which were rich in β6 integrin, less dynamic, more strongly adherent, and turned over less efficiently than the FA of wild-type cells.[18]

Transduction of signals from the integrin platforms to the actin cytoskeleton is perturbed in the absence of kindlin-1, as demonstrated by the fact that KS cells were not able to polarize or respond to extracellular signals by properly modulating the cytoskeleton and building large lamellipodia. Several clinical and cellular manifestations in KS are remarkably similar to deficiencies of Rho GTPases. However, the interpretation of such phenotypic abnormalities observed *in vitro* and *in vivo* is rather complex, since activation of the three main Rho GTPases, Rac1, RhoA, and Cdc42, and their signal transduction pathways are depressed to various degrees, and reciprocal cross talk can influence the system.

Activation of Rac1, which is induced by pathways involving integrins, growth factor receptors, cadherin signaling, or by cross talk with other Rho GTPases,[37] is strongly diminished in KS cells. Our findings show that in the absence of kindlin-1, cells fail to extend a large lamellipodium typical of control keratinocytes and to sense their migration because of failure in Rac1 activation, thus placing kindlin-1 upstream of Rac1 in the signal pathway controlling lamellipodia formation. This is in agreement with other studies showing that integrin-mediated adhesion and $\alpha 3\beta 1$-dependent activation of Rac1 play a role in the formation of polarized, stable lamellipodia in migrating epithelial cells,[38,39] or that a dominant negative Rac1 mutant, Rac1N17, inhibited formation of stable leading lamellipodia.[38] In the epidermis, Rac1 is also involved in maintenance of stem cells[40] and cell-cell contacts.[41] This is presumably reflected by epidermal atrophy in KS skin[14] and by the fact that in the wound-scratch assays KS keratinocytes did not preserve cell-cell contacts and migrate as a front, but moved independently as scattered cells. However, in KS skin and keratinocytes we found new evidence for an abnormal distribution or expression of E-cadherin (Supplementary Figure 2, see *http://ajp.amjpathol.org*). Yet another function of Rac1 was uncovered in a mouse model with keratinocyte-restricted deletion of the *rac1* gene, which revealed that Rac1 is crucial for hair follicle integrity, but not for maintenance of the epidermis.[42] The fact that patients with KS do not have hair abnormalities may either demonstrate a difference between a mouse model and the human system, or be a sign of redundancy. Transgenic mice expressing a dominant inhibitory mutant of Rac under the control of the keratin 14 promotor do not exhibit an obvious skin phenotype,[43] but show protracted wound re-epithelization because the proliferation of wound-edge keratinocytes and the centripetal migration of the neo-epidermis is impaired. Analysis of the cell dynamics of transgenic and control keratinocytes identified decreased lamella-protrusion persistence and increased ruffle frequency,[43] similar to our observations in kindlin-1 deficient keratinocytes.

In mice with keratinocyte-restricted deletion of the *cdc42* gene, the skin basement membrane components

became aberrantly deposited, and the processing of laminin 332 was impaired in parts of the dermal-epidermal junction. These abnormalities grew more severe with age and corresponded to localized absence of the basement membrane in older mutant mice.[44] Intriguingly, these features are very similar to the pathology of KS skin, ie, abnormal deposition of basement membrane components, disorganized architecture of the dermal-epidermal junction, and progression of the phenotype with advancing age,[14,44] and thus support the physiological relevance of kindlin-1 in regulation of cell polarity via Cdc42.

The dissection of signal transduction pathways in KS cells also revealed a role for cofilin, a downstream target of Rac1 and Cdc42, which is involved in regulating actin organization, generation of lamellipodia and determination of the direction of cell motility.[45–47] Two kinase families phosphorylate and thereby deactivate cofilin: the LIM and the TES kinases. The former are activated by phosphorylation through Rho GTPase pathways involving Rac1, Cdc42, and RhoA. The latter, in contrast, are dependent on integrin engagement on cell attachment.[48] Functionally, LIMK and TES kinases promote F-actin stability through cofilin phosphorylation and deactivation.[48] Subsequently, in KS cells reduced LIMK activity leads to increased levels of active cofilin. This correlates well with our video microscopy observations.[6] KS cells migrate slowly in an undirected manner and, concomitantly, exhibit increased protrusion-retraction activity at their periphery, which is associated with an increased actin polymerization—depolymerization turnover and cofilin activity.

RhoA is activated early in keratinocyte differentiation and plays an essential role in establishment of cell–cell adhesion.[49,50] It is involved in the maintenance of desmosomes, and interference with RhoA signaling contributes to pathogenesis of pemphigus, an autoimmune disease with intraepidermal blistering.[51] Defects of cell-cell adhesion or epidermal differentiation are not obvious in KS skin and, therefore, the contribution of reduced RhoA activity to the *in vivo* phenotype is difficult to assess. However, since inhibition of endogenous Rho signaling by itself is sufficient to induce expression of early differentiation markers, like keratin 1 and 10, in cells that maintain active DNA replication,[50] reduced RhoA activation can be predicted to contribute to reduced proliferation capacity of basal keratinocytes and epidermal atrophy in KS.[6]

Taken together, the comparison of signaling events in kindlin-1 deficient and normal human keratinocytes allowed us to discern molecular interactions of kindlin-1 and its role in regulating cellular processes (Figure 8). The *in vitro* and *in vivo* findings implicate kindlin-1 as an important component in integrin-mediated signaling cascades that transduce information from cell surface receptors to focal adhesions and to actin cytoskeleton in epithelial cells. Complex formation with β1 integrin, α-actinin, migfilin, and FAK, and modulation of Rho GTPases give important clues for understanding the role of kindlin-1 in maintenance of cell shape, adhesion to basement membranes, proliferation, and directional migration of keratinocytes. Vice versa, these data shed new light into the pathogenic mechanisms underlying epithelial fragility and atrophy in KS, which manifest as skin blistering, tenacious mucosal erosions, and ulcerative colitis, and allow design of novel molecular therapy strategies.

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